

Family- and Genus-Level 16S rRNA-Targeted Oligonucleotide Probes for Ecological Studies of Methanotrophic Bacteria

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Methanotrophic bacteria play a major role in the global carbon cycle, degrade xenobiotic pollutants, and have the potential for a variety of biotechnological applications. To facilitate ecological studies of these important organisms, we developed a suite of oligonucleotide probes for quantitative analysis of methanotroph-specific 16S rRNA from environmental samples. Two probes target methanotrophs in the family *Methylocystaceae* (type II methanotrophs) as a group. No oligonucleotide signatures that distinguish between the two genera in this family, *Methylocystis* and *Methylosinus*, were identified. Two other probes target, as a single group, a majority of the known methanotrophs belonging to the family *Methylococcaceae* (type I/X methanotrophs). The remaining probes target members of individual genera of the *Methylococcaceae*, including *Methylobacter*, *Methylomonas*, *Methylomicrobium*, *Methylococcus*, and *Methylocaldum*. One of the family-level probes also covers all methanotrophic endosymbionts of marine mollusks for which 16S rRNA sequences have been published. The two known species of the newly described genus *Methylosarcina* gen. nov. are covered by a probe that otherwise targets only members of the closely related genus *Methylomicrobium*. None of the probes covers strains of the newly proposed genera *Methylocella* and “*Methylothermus*,” which are polyphyletic with respect to the recognized methanotrophic families. Empirically determined midpoint dissociation temperatures were 49 to 57°C for all probes. In dot blot screening against RNA from positive- and negative-control strains, the probes were specific to their intended targets. The broad coverage and high degree of specificity of this new suite of probes will provide more detailed, quantitative information about the community structure of methanotrophs in environmental samples than was previously available.

Methanotrophic bacteria are ecologically and technologically important because they comprise a critical link in the global carbon cycle, act as N₂ fixers and ammonia oxidizers, degrade a wide array of organic contaminants, and have biotechnological potential for single-cell protein production and novel enzyme functions (34, 43). Methanotrophs are interesting biologically because they are physiologically and phylogenetically unique. With the exception of two recent isolates (8, 24), all known methanotrophs belong to two monophyletic families: type I/X methanotrophs belong to the family *Methylococcaceae* within the γ -*Proteobacteria*, and type II methanotrophs belong to the family *Methylocystaceae* within the α -*Proteobacteria* (10, 11, 14). For convenience and clarity, we will refer to the former as γ -methanotrophs and to the latter as α -methanotrophs when identifying them phylogenetically. No other phylogenetic clade is known to use CH₄ as a sole C and energy source (34). Hence, methanotrophs provide a striking example of a direct correspondence between physiology and phylogeny, making it possible to link process measurements with molecular phylogenetic approaches *in situ* (15, 17).

Although 16S rRNA-based phylogenies have been used effectively to resolve long-standing confusion over methano-

troph taxonomy (13, 14), a comprehensive suite of 16S rRNA-targeted oligonucleotide probes for the methanotrophs has proven difficult to design (9, 34). Some probes have been useful in monitoring CH₄ enrichment cultures (9, 37) or quantifying undifferentiated groups of diverse methylotrophs, including nonmethanotrophs, in environmental samples (52). However, the probes developed to date either are not specific to methanotrophs (36, 56) or fail to cover a large proportion of known methanotrophs (9, 34). Moreover, due to substantial diversity among the γ -methanotrophs that has been discovered in the past 4 years, such as the genera *Methylosphaera* (12), *Methylocaldum* (7), and *Methylosarcina* (58), many of these organisms have escaped detection by earlier probes.

To facilitate ecological studies of methanotroph communities, we designed a new suite of oligonucleotide probes and optimized them for quantitative hybridization analysis of 16S rRNA from specific groups of methanotrophic bacteria. Our aim was to design a complementary suite of probes that would (i) target methanotrophs to the exclusion of closely related nonmethanotrophic bacteria, (ii) encompass a greater number and wider diversity of known methanotrophic bacteria than achieved previously, and (iii) allow specific detection of methanotrophs at both the family and genus levels.

MATERIALS AND METHODS

Bacterial cultures. The reference cultures used in this study were obtained from various sources, as indicated, and are available from either the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, United Kingdom) or the American Type Culture Collection (ATCC, Manassas, Va.). Ref-

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erence strains include *Methylosinus trichosporium* OB3b (NCIMB 11131) and *Methylococcus capsulatus* Bath (NCIMB 11132) (both provided by J. C. Murrell), *Methylobacter luteus* (NCIMB 11914; provided by R. Knowles), *Methylobacter marinus* A45 (nonextant culture; genomic DNA provided by A. A. DiSpirito), *Methylomicrobium album* BG8 (NCIMB 11123; provided by G. M. King), *Methylomonas rubra* (NCIMB 11913) and *Methylomonas methanica* S1 (NCIMB 11130) (both provided by J. D. Semrau), *Methylocaldum gracile* (NCIMB 11912; purchased from NCIMB), *Caulobacter crescentus* CB15A (ATCC 19089; provided by J. S. Poindexter), and *Escherichia coli* 01:K1(L1):H7 (ATCC 11775; from laboratory stock culture).

All methanotrophs were grown at 30°C, except *Methylococcus capsulatus* Bath and *Methylocaldum gracile*, which were grown at 45°C, in nitrate mineral salts medium with CH₄ and CO₂ at an initial headspace mixing ratio of 45:5:50 (CH₄ to CO₂ to air) (35). *E. coli* was grown in Luria-Bertani broth under standard conditions (53), and *C. crescentus* CB15A was grown in PYCM medium (27) at room temperature.

Sequencing of 16S rRNA genes. Because ambiguous and missing bases in several of the sequences available from GenBank hindered sequence comparisons, we resequenced the 16S rRNA genes of *Methylomonas rubra* NCIMB 11913, *Methylobacter luteus* NCIMB 11914, *Methylomonas methanica* S1 NCIMB 11130, and *Methylobacter marinus* strain A45. Nearly complete (1,450-bp) sequences were obtained for both the sense and antisense strands of the 16S rRNA gene using 5% Long Ranger gel and an ABI PRISM DNA sequencer (41).

Selection of reference sequences. Probes were designed based on reference 16S rRNA sequences available from GenBank (6) and the Ribosomal Database Project (RDP-II) (42), as well as resequencing of key laboratory strains (see Table 1 and Fig. 3). BLAST (GenBank) and Probe Match (RDP-II) database searches were used to assess the potential breadth and specificity of the probe sequences. The reference sequences were aligned with the probe sequences to determine the apparent range of coverage of the candidate probes relative to the abundance and diversity of known methanotrophs. The 16S rRNA sequences specified by accession numbers in Fig. 2 and 3 represent all those available in the databases for confirmed methanotrophic isolates at the time of analysis. With the exception of the methanotrophic endosymbionts of marine mollusks (see below), we did not include sequences obtained from cultures that had not been characterized phenotypically or that were obtained by PCR amplification of environmental DNA.

Only cultured isolates with published, genus-level phylogenetic data were assigned genus designations in Fig. 2 and 3. Three general groups of confirmed methanotrophs were placed under "other α -methanotrophs" (Fig. 2) or "other γ -methanotrophs" (Fig. 3): (i) strains clearly belonging to the α - or γ -methanotrophs, but lacking or having dubious generic affiliations because of insufficient phylogenetic and taxonomic information (for example, "*Methylomonas methanica*" strain 81Z is clearly a γ -methanotroph [57] but has not been characterized at the genus level); (ii) isolates validly assigned to the genera *Methylocella*, *Methylophaera*, and *Methylosarcina*, for which we did not design genus-level probes because there were only one or two known representatives of each genus; and (iii) the methanotrophic endosymbionts of marine mollusks, which lack generic descriptions. Although uncultured, the mollusk endosymbionts were included because they are of active interest to microbial ecologists and evolutionary biologists and because there is strong phenotypic and phylogenetic evidence that they are γ -methanotrophs (19, 20, 25, 28).

All available methanotroph 16S rRNA sequences that met the criteria given above were included in our analysis, regardless of sequence quality. However, a number of sequences appeared to be affected by common sequencing errors, including transposition of bases and duplicated or omitted bases. Some errors could be confirmed because they violated the integrity of the secondary structure of the 16S rRNA molecule, but others could not because they occurred in unpaired loop positions. Because sequence errors make designing group-level probes very difficult, we developed specific criteria for disregarding unexpected mismatches between a probe and a target sequence. We deemed destabilization of secondary structure sufficient grounds for disregarding mismatches. Additionally, we considered any two of the following criteria sufficient: (i) the mismatch occurs in a low-quality sequence as indicated by ambiguous bases in >0.5% of the positions in the entire sequence; (ii) the mismatch results from an ambiguous or missing base in the probe target region; (iii) multiple sequences for the same strain disagree in the mismatch position, and the higher-quality sequence, as indicated by percent ambiguity, matches the probe; (iv) a multiple alignment of all available sequences representing the target group shows that the mismatch is not representative of the target group; (v) the mismatch occurs in a highly conserved position of the 16S rRNA molecule; (vi) the mismatch is consistent with a common sequencing error, such as the transposition of two bases or the repetition of the same base, that disagrees with several other related sequences.

Oligonucleotide probe design. The oligonucleotide probes developed and/or optimized in this study are described in Table 1. The numbering used in probe designation represents the forward position of the homologous base in the *E. coli* 16S rRNA gene. By use of the SEQLAB sequence editor in the Wisconsin Package (Genetics Computer Group, Madison, Wis.), 16S rRNA sequences (>1,300 bp) were aligned initially using the PILEUP function within the editor and then adjusted manually with secondary-structure considerations as described previously (2). With the help of computer-generated consensus sequences, the alignments were scanned visually for signature sequences of 18 to 30 nucleotides that distinguished methanotrophs at the family or genus level. Candidate oligonucleotide sequences were then examined for specificity using the basic BLAST search and Probe Match functions of GenBank and the RDP-II, respectively (5, 42). Except as described below, only sequences exhibiting high specificity for methanotrophs and retrieving a majority of the sequences in their target groups were pursued further.

***T_d* determination and specificity testing.** Oligodeoxynucleotides were synthesized commercially (DNAgency, Malvern, Pa.). Each probe was characterized by empirical determination of its midpoint dissociation temperature (*T_d*) using a serial washing procedure with progressively higher temperatures in a PCR thermal cycler as described by Gullledge and Cavanaugh (32). All *T_d* curves were determined using triplicate blots for both positive and negative controls (see Fig. 1).

The ability of each probe to distinguish between positive and negative controls was screened in Northern dot blot hybridization assays, as described below, using total RNA from reference cultures representing target strains as positive controls and total RNA from reference cultures representing nontarget strains with 1- or 2-base mismatches as negative controls. In all but two cases, a strain with a single-base mismatch with the probe was used as a negative control (Table 1). Because no nontarget organisms that had fewer than two mismatches with probe Am445 were identified, an organism with two mismatches was used as a negative control. Also, because no potential control organisms with fewer than four mismatches to probe Mcd77 were identified, we designed a probe with a single mismatch at position 15 to serve as a negative control (Table 1).

RNA extraction from bacterial cultures. Pure cultures were grown to late-log phase in 40 ml of liquid growth medium and centrifuged at $5,000 \times g$ for 10 min at 4°C. Total RNA was extracted selectively from cell pellets using the FastPrep bead beater system with the FastRNA Blue kit according to the manufacturer's protocol (Bio 101, La Jolla, Calif.). Cells were beaten in the FP120 bead beater for 25 to 40 s at a speed of 6 m/s. After extraction and centrifugation, the RNA pellets were air dried, resuspended in diethyl pyrocarbonate-treated H₂O, and stored at -80°C.

RNA dot blotting and hybridization. Northern dot blots were prepared from RNA extracts as described previously (48) using a Minifold I Microsample Filtration Manifold (Schleicher & Schuell, Keene, N.H.). Blots were prepared with 100 ng of 16S rRNA per dot to be blotted, assuming that 16S rRNA represented 27% of total RNA (47), as described previously (49).

Oligonucleotide probes were labeled enzymatically with ³²P (49), and hybridization assays were carried out as described previously (48). Labeled oligonucleotides were hybridized to the dot blots overnight at 30°C, finishing with two 30-min rinses at the appropriate *T_d* for each probe (Table 1). Oligonucleotide labeling of the dot blots was analyzed by radiodensitometry using a BAS-MS 2025 imaging plate and a Fujix 2000 PhosphorImager, with MacBAS, version 2.5, image analysis software (Fuji Medical Systems, Stamford, Conn.).

Nucleotide sequence accession numbers. The new sequences of the 16S rRNA genes of *Methylomonas rubra* NCIMB 11913, *Methylobacter luteus* NCIMB 11914, *Methylomonas methanica* S1 NCIMB 11130, and *Methylobacter marinus* strain A45 have been deposited in GenBank (accession numbers AF304194 to AF304197).

RESULTS AND DISCUSSION

Overview. In recent years, interest in the physiology, ecology, and evolution of methanotrophs has intensified, and there is high demand for tools to facilitate quantitative studies of in situ methanotroph community structure (21, 34, 46, 50). Our objectives were to develop phylogenetic oligonucleotide probes for analysis of methanotrophs at the family and genus levels and to optimize the probes for use in quantitative hybridization through empirical determination of their *T_d*s under standard hybridization conditions.

Visual comparison of aligned 16S rRNA reference sequences initially revealed 36 potential probe sequences for fur-

group	Species/Strain	Phylogeny Reference	GenBank Accession number	%Ambiguity	Probes	
					Am445	Am976
Methylocystis	<i>parvus</i> str. OBBP NCIMB 11129	(13)	Y18945	0.0	ns	
	<i>parvus</i> str. OBBP NCIMB 11129	(13)	AF150805	0.0		
	<i>parvus</i> str. OBBP NCIMB 11129	(13)	M29026	2.6		
	<i>echinoides</i> NCIMB 13100	(33)	L20848	1.1		
	<i>pyriformis</i> NCIMB 13102	(33)	L20803	1.9		
	<i>minimus</i> NCIMB 13099	(33)	L20844	0.1		
	sp. str. LW5	(22)	AF150790	0.0		
	sp. str. EB-1	(33)	AB015608	0.0		
	sp. str. W1 14	(31)	AF153281	0.0		
	sp. str. M	(45)	U81595	0.0		
Methylosinus	<i>trichosporium</i> str. OB3b NCIMB 11131	(13)	Y18947	0.0	1	
	<i>trichosporium</i> str. OB3b NCIMB 11131	(13)	M29024	2.6		
	<i>trichosporium</i> str. OB3b NCIMB 11131	(13)	AF150804	0.0		
	<i>sporium</i> NCIMB 11126	(13)	Y18946	0.0		
	<i>sporium</i> NCIMB 11126	(13)	M95665	0.0		
	<i>methanica</i> str. 81Z (formerly "Methylosporovibrio")	(34)	M29025	2.8		
	sp. str. PW1	(22)	AF150802	0.0		
	sp. str. LW2	(22)	AF150786	0.0		
	sp. str. LW3	(22)	AF150788	0.0		
	sp. str. LAC NCIMB 13214	(33)	M95664	0.0		
other Am	sp. str. B NCIMB 13103	(33)	M95663	0.2	2	
	str. IMV-B 3060	(59)	L20845	0.0		
	str. LR1	(26)	Y18442	0.0		
	str. AML-A3	(59)	AF177298	0.0		
	str. AML-A6	(59)	AF177299	0.0		
non-Am	<i>Methylocella palustris</i> str. K ATCC 700799 ^T	(24)	Y17144	0.0	4	1
	<i>Caulobacter crescentus</i> str. CB15A	(1)	AJ227757	0.0	2	1
	<i>Afpia</i> genosp. 9 str. G8993	(16)	U87780	0.0	7	
	<i>Bosea thiooxidans</i> str. BL-42 ^T	(23)	X81044	0.0	6	
	<i>Rhodospseudomonas acidiphila</i>	(57)	M34128	2.5	7	

FIG. 2. Range of strain coverage for oligonucleotide probes targeting α -methanotrophs (Am). % Ambiguity, percentage of positions within the entire sequence that indicate ambiguous bases, shown as an index of overall sequence quality. Under "Probes," solid fill indicates identity between the probe and a target sequence; cross-hatching indicates identity between the probe and a nonmethanotroph; numbers are numbers of mismatches between the probe sequence and the corresponding 16S rRNA sequence. Where a number is shown in white on a solid background, the apparent mismatches were disregarded based on criteria outlined in Materials and Methods. An open diamond denotes the occurrence of one or more ambiguous bases in the probe target region that are consistent with the probe sequence. For example, if the probe has an A corresponding to a Y (International Union of Pure and Applied Chemistry [IUPAC] ambiguity code for C or T), then the possible T is consistent with the probe sequence. ns, no sequence available in probe target region.

two family-level probes (Gm633 and Gm705) covered 82% of the available γ -methanotroph 16S rRNA sequences (Fig. 3). Gm705 had the broadest coverage, including representatives of six γ -methanotroph genera and the methanotrophic endosymbionts of marine mollusks. Gm633 was more limited, but it provided better coverage of *Methylobacter* and *Methylomicrobium* spp. The genera *Methylocaldum* and *Methylosarcina* eluded these two probes. However, almost complete coverage of the family can be achieved by combining these family-level probes with two or more of the genus-level probes described below.

Several probes provide genus-level detection of the closely related γ -methanotroph genera *Methylobacter*, *Methylomicrobium*, and *Methylomonas* (Fig. 3). Together, probes Mlb482 and Mlb662 covered all representatives of the genus *Methylobacter*. An indicated 6-base mismatch between Mlb482 and the 16S rRNA sequence for *Methylobacter* sp. strain T20 (AF131868) stems from seemingly errant insertions at positions 497 and 505 (*E. coli* numbering), as judged by the level of within-genus sequence conservation in the probe region and the fact that the indicated base change would violate the secondary structure of the 16S rRNA molecule. If the two apparent insertions are

disregarded, the sequence matches Mlb482 perfectly. Probes Mmb482 and Mmb1007 each matched all available *Methylomicrobium* sequences. Mmb1007 also covered both strains of the newly described genus *Methylosarcina*, which are closely related to *Methylomicrobium* spp. (58). Three other probes covered all of the recognized *Methylomonas* isolates. Representatives of this genus fell into two groups that differ by an A versus a C at position 746 (*E. coli* numbering). We designed two probes (Mlm732a and Mlm732b) to distinguish between the two subgenus groups. Mlm482 provided the broadest coverage of *Methylomonas* spp., but all representatives of the genus were covered only when the three Mlm probes were combined.

Three probes covered all representatives of the two recognized thermophilic genera, *Methylococcus* and *Methylocaldum*. Mlc123 and Mlc1436 each matched all *Methylococcus* sequences available. PCR primers corresponding to these two probes might be ideal for specific amplification of nearly complete (~1,300-bp) 16S rRNA genes from *Methylococcus* strains in environmental samples. Probe Mcd77 covered the three recognized strains of the recently described genus *Methylocaldum*. The target region was unique, and a Probe Match analysis retrieved no sequences with fewer than four mismatches from non-*Methylocaldum* species.

The complete suite of γ -methanotroph probes covered 97% of the strains listed in Fig. 3; only two sequences were not covered. One is that of *Methylomonas methanica* strain 81Z, cultures of which are no longer extant and whose affiliation with the genus *Methylomonas* was never verified (J. P. Bowman and P. N. Green, personal communication). Because this sequence is of low overall quality (3.3% ambiguity), one or more of the indicated mismatches could be incorrect. The other organism not covered by the probes is a novel thermophilic methanotroph, "*Methylothermus*" sp. strain HB. Because it is the only known γ -methanotroph that is polyphyletic with respect to the family *Methylococcaceae* (8), this result was expected.

Probe specificity and optimization for quantitative hybridization. The probes described here are intended to quantify 16S rRNA from specific microbial populations against a background of many unknown populations in environmental samples. The probes must discriminate against unknown, nontarget 16S rRNA that may have a difference of only 1 base from the intended target. The primary factor for achieving stringent specificity and quantitative hybridization of 16S rRNA from environmental samples is accurate determination of the melting characteristics of the probe-target duplex. Hence, empirical determination of the T_d is essential (32, 54). We have optimized the probes presented here for stringent discrimination against nontarget RNA and also for quantitative hybridization by empirically determining the T_d for each probe.

The T_d s of the probes ranged from 49 to 57°C (Table 1). When Northern blots were hybridized overnight and then washed at the appropriate T_d , target and nontarget rRNAs were visually distinguishable on blots and yielded quantitatively distinct results when analyzed using a scintillation counter (Fig. 1) or a phosphorimager (data not shown). These results verify that the use of known concentrations of reference rRNA as standards will permit quantitative analysis of environmental rRNA possessing the target sequence, as demonstrated previously (49, 54).

Group	Species/Strain	Phylogeny Reference	GenBank Accession number	%Ambiguity	Probes												
					Gm633	Gm705	Mlb482	Mlb662	Mmb482	Mmb1007	Mlm482	Mlm732a	Mlm732b	Mlc123	Mlc1436	Mcd77	
Methylobacter	<i>luteus</i> NCIMB 11914 [†]	(13, 14)	M95657	0.0					1	1	1	1	1	1	1	1	
	<i>luteus</i> NCIMB 11914 [†]	(13, 14)	X72772	0.0			1		1	1	1	1	1	1	1	1	
	<i>luteus</i> NCIMB 11914 [†]	(13, 14)	AF304195	0.0					1	1	1	1	1	1	1	1	
	<i>luteus</i> formerly 'bovis' str. 89	(13, 14)	L20839	0.8	♦	1			1	1	1	1	1	1	1	1	
	<i>whittenburyi</i> (formerly 'vinlandii') str. 87	(13, 14)	L20841	0.7	♦	3			1	1	1	1	1	1	1	1	
	<i>whittenburyi</i> (formerly 'capsulatus') NCIMB 11128 [†]	(13, 14)	L20843	0.7	♦	1			1	1	1	1	1	1	1	1	
	<i>whittenburyi</i> (formerly 'capsulatus') NCIMB 11128 [†]	(13, 14)	X72773	0.0					1	1	1	1	1	1	1	1	
	<i>marinus</i> str. A4 (formerly <i>Methylomonas</i> ; nonextant)	(13, 14)	M95658	6.8					1	1	1	1	1	1	1	1	
	<i>marinus</i> str. A45 (formerly <i>Methylomonas</i> ; nonextant)	(13, 14)	AF304197	0.0					1	1	1	1	1	1	1	1	
	sp. str. BB5.1 (nonextant)	(22)	AF016981	0.1					1	1	1	1	1	1	1	1	
	sp. str. LW1	(22)	AF150784	0.0					1	1	1	1	1	1	1	1	
	<i>psychrophilus</i> str. Z-0021	(55)	AF152597	0.0	4	2		1	1	2	2	1	2	2	3	ns	
sp. str. T20	(51)	AF131868	0.0	4	1	6	1	1	2	1	2	2	3	3	5		
Methylostrombium	<i>album</i> str. BG8 NCIMB 11123 [†]	(13, 14)	M95659	7.7	2	1	3	4	2		3	4	3	5	ns	>8	
	<i>album</i> str. BG8 NCIMB 11123 [†]	(13, 14)	X72777	0.1		1	1				1	3	2	2	5	>8	
	<i>agile</i> str. A30 NCIMB 11124 [†]	(13, 14)	X72767	0.2			1	2			1	4	2	2	5	>8	
	<i>pelagicum</i> NCIMB 2265 [†]	(13, 14)	X72775	0.1			1	1			1	2	3	2	6	>8	
	<i>pelagicum</i> NCIMB 2265 [†]	(13, 14)	U05570	0.0		1	1	2			1	2	3	2	ns	>8	
	<i>pelagicum</i> NCIMB 2265 [†]	(13, 14)	1.35540	0.0		1	1	2			1	2	3	2	ns	>8	
	sp. str. NI	(29)	D89279	0.0		1	1	2			1	2	3	2	ns	>8	
	<i>alcaliphilum</i> str. 5z NCIMB 13566	(40)	AF096091	0.0		1	1	2			1	2	3	2	ns	>8	
	<i>buryaticum</i> str. 5G	(39)	AF096092	0.0	2	1	1	2			1	2	3	2	ns	>8	
	<i>buryaticum</i> str. 7G	(39)	AF096093	0.0	2	1	1	2			1	2	3	2	ns	>8	
	Methylomonas	<i>aurantiaca</i> str. JB103 ACM 3406 [†]	(13, 14)	X72776	0.1	1		2	1	1	1	4		2	1	3	7
		<i>iodinarum</i> str. JB13 ACM 3268 [†]	(13, 14)	X72778	0.2	1		2	1	1	1	4		2	1	3	7
sp. str. LW19		(22)	AF150798	0.0	2		2	1	1	6		2	1	4	5	>8	
sp. str. LW21		(22)	AF150800	0.0	2		2	1	1	6		2	1	4	5	>8	
<i>methanica</i> str. S1 NCIMB 11130 [†]		(13, 14)	L20840	1.8	1		2	2	1	6		1	1	4	5	>8	
<i>methanica</i> str. S1 NCIMB 11130 [†]		(13, 14)	*	0.9	1		1	1	1	4		1	1	4	5	>8	
<i>methanica</i> str. S1 NCIMB 11130 [†]		(13, 14)	AF150806	0.0	1		1	1	1	3		1	1	4	5	>8	
<i>methanica</i> str. S1 NCIMB 11130 [†]		(13, 14)	AF304196	0.0	1		2	1	1	5		1	3	6	>8	>8	
sp. str. KSP11		(33)	AB015604	0.4	1		2	1	1	4		1	4	ns	>8	>8	
sp. str. KSP11		(33)	AB015603	0.0	1		2	1	1	4		1	4	5	>8	>8	
sp. str. KSW11		(33)	AB015602	0.7	1		2	1	1	4		1	4	5	>8	>8	
sp. str. LW13		(22)	AF150792	0.0	1		2	1	1	4		1	4	5	>8	>8	
sp. str. LW15		(22)	AF150794	0.0	1		2	1	1	4		1	4	5	>8	>8	
sp. str. LW16		(22)	AF150796	0.0	1		2	1	1	6		1	4	5	>8	>8	
<i>scandinavica</i> str. SR-5		(38)	AJ131369	0.9	2		2	1	1	2		1	4	ns	>8	>8	
<i>'rubra'</i> NCIMB 11913		(13, 14)	AF150807	0.0	2		2	1	1	2		1	4	5	>8	>8	
<i>'rubra'</i> NCIMB 11913		(13, 14)	M95662	0.8	2		1	1	1	2		1	1	5	6	>8	
<i>'rubra'</i> NCIMB 11913		(13, 14)	AF304194	0.0	2		2	1	1	2		1	1	4	5	>8	
sp. str. 761H NCIMB 11931		(14)	L20846	0.6	1		3	2	2	8		1	1	4	8	>8	
Mlc	<i>thermophilus</i> str. IMV-B 3037	(13, 14)	X73819	0.2	5		3	3	3	8		4	3	3	>8	>8	
	sp. str. JB140	(13, 14)	X72769	0.1	4		3	3	3	8		4	2	3	>8	>8	
	<i>capsulatus</i> str. Texas [†] NCIMB 11853 [†]	(13, 14)	X72770	0.1	6		3	3	3	8		4	2	3	>8	>8	
	<i>capsulatus</i> str. Bath NCIMB 11132	(13, 14)	X72771	0.0	5		3	3	3	8		4	2	3	>8	>8	
	<i>capsulatus</i> str. Bath NCIMB 11132	(13, 14)	L20842	3.1	5	2	4	2	4	>8	5	4	5	>8	>8	>8	
	<i>capsulatus</i> str. Bath NCIMB 11132	(13, 14)	M29023	3.2	7	3	4	ns	4	>8	5	3	4	♦	ns	>8	
Mcd	<i>szegediense</i> str. OR2 [†]	(7, 8)	U89300	0.1	4	1	4	3	4	5	5	3	4	1	5	>8	
	<i>tepidum</i> str. LK6 [†]	(7, 8)	U89297	0.2	4	1	4	3	4	5	5	3	4	1	5	>8	
	<i>gracile</i> str. VKM-14L NCIMB 11912 [†]	(7, 8)	U89298	0.3	4	1	4	3	4	6	5	3	4	1	5	>8	
other Gm	<i>Methylosarcina fibrata</i> str. AML-C10 [†] ATCC 700909 [†]	(58)	AF177296	0.0	4	1	3	2	2		3	2	3	2	4	>8	
	<i>Methylosarcina quisquiliarum</i> str. AML-D4 [†] ATCC 700908 [†]	(58)	AF177297	0.0	4	1	3	2	2		3	2	3	2	5	>8	
	<i>Methylosphaera hansonii</i> str. AM6 [†] ACM 549 [†]	(12)	U67929	0.1	5		3	1	2	3	3	4	3	4	4	>8	
	<i>Methylosphaera hansonii</i> str. AM11	(12)	U77533	0.1	5		3	1	2	3	3	4	3	4	4	>8	
	<i>Bathymodiolus japonicus</i> gill endosymbiont	(28)	AB036711	0.0	2		1	2	2	2	1	2	2	3	6	>8	
	<i>Bathymodiolus childressi</i> gill endosymbiont	(25)	U05595	0.0	5		1	2	2	2	1	2	2	3	ns	>8	
	<i>Bathymodiolus puteoserpentis</i> gill endosymbiont	(25)	U29164	0.0	4		1	2	2	2	2	2	3	3	ns	>8	
	<i>Bathymodiolus platifrons</i> gill endosymbiont	(28)	AB036710	0.0	2		1	2	2	2	2	2	2	3	6	>8	
	<i>'Methylomonas methanica'</i> str. 81Z (nonextant)	(57)	M29022	3.3	2	4	5	>8	4	5	3	5	5	2	ns	>8	
	<i>'Methylothermus'</i> sp. str. HB	(8)	U89299	0.5	3	4	4	4	4	>8	5	5	5	5	7	>8	
	non-Gm	<i>Cycloclasticus oligotrophus</i>	(18)	AF148215	0.1	4	1	3				2	>8	3	3	4	5
		<i>Cycloclasticus puegeti</i> PS-1	(30)	L34955	0.1	4	1	3				2	>8	3	3	4	5
<i>Cycloclasticus</i> sp. str. N3-PA321		(30)	U57920	0.1	4	1	3				2	>8	3	3	4	5	
<i>Cycloclasticus</i> sp. str. G		(30)	AF093002	0.1	4	1	3				2	>8	3	3	4	5	

FIG. 3. Range of strain coverage for oligonucleotide probes targeting γ -methanotrophs (Gm), *Mlc*, *Methylococcus*; *Mcd*, *Methylocaldum*. % Ambiguity, percentage of positions within the entire sequence that indicate ambiguous bases, shown as an index of overall sequence quality. The unpublished 16S rRNA sequence for *Methylomonas methanica* strain S1 (marked with a star in the "GenBank accession number" column) is available as RDP sequence Mlm.metha1 (C. R. Woese, 1991). Under "Probes," solid fill indicates identity between the probe and a target sequence; diagonal hatching indicates identity between the probe and a nontarget γ -methanotroph strain; cross-hatching indicates identity between the probe and a nonmethanotroph; numbers are numbers of mismatches between the probe sequence and the corresponding 16S rRNA sequence. Where a number is shown in white on a solid background, the apparent mismatches were disregarded based on criteria outlined in Materials and Methods. An open diamond denotes the occurrence of one or more ambiguous bases in the probe target region that are consistent with the probe sequence. For example, if the probe has an A corresponding to a Y (IUPAC ambiguity code for C or T), then the possible T is consistent with the probe sequence. ns, no sequence available in probe target region.

Probes Am445, Mmb1007, Mlm482, Mlm732b, Mlc123, and Mcd77 each exhibited at least two base mismatches against any nonmethanotroph sequence, whereas probes Gm633, Gm705, Mlb482, Mmb482, Mlm732a, and Mlc1436 each exhibited at least one base mismatch with any nonmethanotroph sequence. Probes Mlb662, Mmb482, and Mmb1007 matched sequences from one to four γ -methanotrophs outside their respective target genera (Fig. 3). Although we consider this problem to be minor, these probes could yield ambiguous results for fine-scale descriptions of γ -methanotroph communities. All other

genus-level probes were specific to their intended target genera. The α - and γ -methanotroph probes had no cross-family hybridization potential.

Two probes, Am976 and Mlb662, present the more serious problem of complementing 16S rRNA sequences of some nonmethanotrophic bacteria. They have been retained despite this weakness for two reasons. First, they are needed to ensure complete coverage of their target groups, in combination with other probes, when broad-spectrum probing is desired. Second, they were deemed particularly useful for certain experi-

TABLE 2. Results from probing the GenBank database^a

Source of matching sequence	No. of identical sequences retrieved for each probe													
	Am445	Am976	Gm633	Gm705	Mlb482	Mlb662	Mmb482	Mmb1007	Mlm482	Mlm732a	Mlm732b	Mlc123	Mlc1436	Mcd77
Methanotrophic isolates	36	26	16	33	8	11	13	13	14	7	4	5	6	3
Putative methanotrophic clone from environmental samples	33	5	8	24	22	9	1	3	7	3	0	0	0	0
Nonmethanotrophic cultures and environmental clones	0	19 ^b	0	0	0	7 ^c	0	0	0	0	0	0	7 ^d	0
Unidentified isolates and environmental clones	7	15	0	1	19	0	0	0	3	1	0	0	1	0
Total sequences retrieved	76	65	24	58	49	27	14	16	24	11	4	5	14	3

^a Searches were performed on 6 March 2001.

^b Of 19 nonmethanotroph sequences, 16 represent *Afipia* and *Bosea* sequences.

^c All nonmethanotroph sequences represent *Cycloclasticus* strains.

^d Of seven nonmethanotroph sequences, six are closely related environmental clones from putative β -*Proteobacteria* retrieved from an activated sludge reactor.

mental approaches, such as monitoring of CH₄ enrichment cultures, use as PCR primers in cases where amplified products are to be sequenced for identification, or analysis of community composition in environmental samples where the nontarget organisms with which the probes hybridize should be minor components of the community. For instance, because marine *Cycloclasticus* spp. were the only nonmethanotrophs that matched Mlb662 (Fig. 3), this probe might be appropriate for probing nonmarine samples.

Probing the database. The GenBank database contains thousands of bacterial 16S rRNA gene sequences from cultures and environmental clones (6). Hence, "probing" this database should provide a powerful assessment of a probe's ability to select specifically for methanotroph sequences against a background of myriad nonmethanotroph sequences. We subjected each probe sequence to a basic BLAST search (5) and examined sequences retrieved with an identical match. Only sequences identified as 16S rRNA genes were considered. The organism identifications were based solely on information provided in the accession records or in publications cited therein.

Eleven of 14 probes retrieved only sequences that were identified as methanotrophs (Table 2). Probes Am976, Mlb662, and Mlc1436 retrieved a number of sequences representing a narrow range of nonmethanotrophic taxa. The first two of these probes matched environmentally restricted taxa, such as obligate pathogens (*Afipia* spp.) and obligate marine bacteria (*Cycloclasticus* spp.). If used strategically, therefore, these probes are likely to be useful for studying methanotroph communities. From the data in Table 2, it would be premature to conclude that Mlc1436 is nonspecific. All but one of the nonmethanotroph sequences retrieved by this probe were nearly identical clones of putative β -*Proteobacteria* from an activated sludge reactor. However, no cultured organisms belonging to the β -*Proteobacteria* were retrieved, and no published data were cited in the accession records to confirm the phylogenetic position of these environmental clones. Overall, the data in Table 2 suggest that at least 11 and possibly 12 of the probes presented here are highly specific to methanotrophic bacteria and that the two clearly nonspecific probes should hybridize to a phylogenetically limited range of nonmethanotrophs with restricted environmental distributions.

Summary and conclusions. The breadth and specificity of the probes reported here are unprecedented, providing 97% coverage of the 87 methanotroph 16S rRNA sequences exam-

ined (Fig. 2 and 3). Several new methanotroph genera that have been proposed recently following the isolation of novel strains are covered. Of the three strains apparently not covered by the probes, one is no longer extant and the available 16S rRNA sequence is of low overall quality, bringing into question whether the indicated probe mismatches are correct. The other two strains (*Methylocella palustris* sp. strain K and *Methylothermus* sp. strain HB) that did not match any probe are polyphyletic with respect to the *Methylocystaceae* and *Methylococcaceae*, thus reflecting the high specificity of the probes to the phylogenetic clades they were designed to target. Initial results from studies with several soils indicate that the probes are effective for studying methanotroph communities in soil (unpublished data), perhaps the most difficult substrate on which to perform quantitative hybridization assays (4). Hence, all of the methanotroph taxa that have become well known through years of laboratory studies, as well as several recently described taxa, can now be studied at both the family and genus levels in environmental samples by using the probes reported here.

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REFERENCES

- Abraham, W. R., C. Strömpl, H. Meyer, S. Lindholm, E. R. Moore, R. Christ, M. Vancanneyt, B. J. Tindall, A. Bennisar, J. Smit, and M. Tesar. 1999. Phylogeny and polyphasic taxonomy of *Caulobacter* species. Proposal of *Maricaulis* gen. nov. with *Maricaulis maris* (Poindexter) comb. nov. as the type species, and emended description of the genera *Brevundimonas* and *Caulobacter*. Int. J. Syst. Bacteriol. 49:1053-1073.
- Ahmad, A., J. P. Barry, and D. C. Nelson. 1999. Phylogenetic affinity of a wide, vacuolate, nitrate-accumulating *Beggiatoa* sp. from Monterey Canyon, California, with *Thioploca* spp. Appl. Environ. Microbiol. 65:270-277.
- Alm, E. W., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin. 1996. The Oligonucleotide Probe Database. Appl. Environ. Microbiol. 62:3557-3559.
- Alm, E. W., D. D. Zheng, and L. Raskin. 2000. The presence of humic substances and DNA in RNA extracts affects hybridization results. Appl. Environ. Microbiol. 66:4547-4554.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990.

- Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
6. Benson, D. A., D. J. Lipman, I. Karsch-Mizrachi, J. Ostell, B. A. Rapp, and D. L. Wheeler. 2000. GenBank. *Nucleic Acids Res.* **28**:15–18.
 7. Bodrossy, L., E. M. Holmes, A. J. Holmes, K. L. Kovacs, and J. C. Murrell. 1997. Analysis of 16S rRNA and methane monooxygenase gene sequences reveals a novel group of thermotolerant and thermophilic methanotrophs, *Methylocaldum* gen. nov. *Arch. Microbiol.* **168**:493–503.
 8. Bodrossy, L., K. L. Kovacs, I. R. McDonald, and J. C. Murrell. 1999. A novel thermophilic methane-oxidising *γ-Proteobacterium*. *FEMS Microbiol. Lett.* **170**:335–341.
 9. Bourne, D. G., A. J. Holmes, N. Iversen, and J. C. Murrell. 2000. Fluorescent oligonucleotide rDNA probes for specific detection of methane oxidising bacteria. *FEMS Microbiol. Ecol.* **31**:29–38.
 10. Bowman, J. P. Family *Methylocystaceae* fam. nov. In G. M. Garrity (ed.), *Bergey's manual of systematic bacteriology*, 2nd ed., vol. 2, in press. Springer-Verlag, New York, N.Y.
 11. Bowman, J. P. Order VI. *Methylococcales* ord. nov. In G. M. Garrity (ed.), *Bergey's manual of systematic bacteriology*, 2nd ed., vol. 2, in press. Springer-Verlag, New York, N.Y.
 12. Bowman, J. P., S. A. McCammon, and J. H. Skerratt. 1997. *Methylosphaera hansonii* gen. nov., sp. nov., a psychrophilic, group I methanotroph from Antarctic marine-salinity, meromictic lakes. *Microbiology* **143**:1451–1459.
 13. Bowman, J. P., L. I. Sly, P. D. Nichols, and A. C. Hayward. 1993. Revised taxonomy of the methanotrophs—description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. *Int. J. Syst. Bacteriol.* **43**:735–753.
 14. Bowman, J. P., L. I. Sly, and E. Stackebrandt. 1995. The phylogenetic position of the family *Methylococcaceae*. *Int. J. Syst. Bacteriol.* **45**:182–185.
 15. Bratina, B. J., G. A. Brusseau, and R. S. Hanson. 1992. Use of 16S rRNA analysis to investigate phylogeny of methylotrophic bacteria. *Int. J. Syst. Bacteriol.* **42**:645–648.
 16. Brenner, D. J., D. G. Hollis, C. W. Moss, C. K. English, G. S. Hall, J. Vincent, J. Radošević, K. A. Birkness, W. F. Bibb, F. D. Quinn, B. Swaminathan, A. E. Weaver, M. W. Reeves, S. P. O'Connor, P. S. Hayes, F. C. Tenover, A. G. Steigerwalt, B. A. Perkins, M. I. Daneshvar, B. C. Hill, J. A. Washington, T. C. Woods, S. B. Hunter, T. L. Hadfield, G. W. Ajello, A. F. Kaufmann, D. J. Wear, and J. D. Wenger. 1991. Proposal of *Afpia* gen. nov., with *Afpia felis* sp. nov. (formerly the cat scratch disease bacillus), *Afpia clevelandensis* sp. nov. (formerly the Cleveland Clinic Foundation strain), *Afpia broomeae* sp. nov., and three unnamed genospecies. *J. Clin. Microbiol.* **29**:2450–2460.
 17. Brusseau, G. A., E. S. Bulygina, and R. S. Hanson. 1994. Phylogenetic analysis and development of probes for differentiating methylotrophic bacteria. *Appl. Environ. Microbiol.* **60**:626–636.
 18. Button, D. K., B. R. Robertson, P. W. Lepp, and T. M. Schmidt. 1998. A small, dilute-cytoplasm, high-affinity, novel bacterium isolated by extinction culture and having kinetic constants compatible with growth at ambient concentrations of dissolved nutrients in seawater. *Appl. Environ. Microbiol.* **64**:4467–4476.
 19. Cavanaugh, C. M., P. R. Levering, J. S. Maki, R. Mitchell, and M. Lidstrom. 1987. Symbiosis of methylotrophic bacteria and deep-sea mussels. *Nature* **325**:346–348.
 20. Childress, J. J., C. R. Fisher, J. M. Brooks, M. C. Kennicutt II, R. Bidigare, and A. E. Anderson. 1986. A methanotrophic marine molluscan (*Bivalvia*, *Mytilidae*) symbiosis: mussels fueled by gas. *Science* **233**:1306–1308.
 21. Conrad, R. 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2 , CO , CH_4 , OCS , N_2O , and NO). *Microbiol. Rev.* **60**:609–640.
 22. Costello, A. M., and M. E. Lidstrom. 1999. Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Appl. Environ. Microbiol.* **65**:5066–5074.
 23. Das, S. K., A. K. Mishra, B. J. Tindall, F. A. Rainey, and E. Stackebrandt. 1996. Oxidation of thiosulfate by a new bacterium, *Bosea thiooxidans* (strain BI-42) gen. nov., sp. nov.: analysis of phylogeny based on chemotaxonomy and 16S ribosomal DNA sequencing. *Int. J. Syst. Bacteriol.* **46**:981–987.
 24. Dedysh, S. N., W. Liesack, V. N. Khmelenina, N. E. Suzina, Y. A. Trotsenko, J. D. Semrau, A. M. Bares, N. S. Panikov, and J. M. Tiedje. 2000. *Methylocella palustris* gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. *Int. J. Syst. Evol. Microbiol.* **50**:955–969.
 25. Distel, D. L., H. K. Lee, and C. M. Cavanaugh. 1995. Intracellular coexistence of methano- and thioautotrophic bacteria in a hydrothermal vent mussel. *Proc. Natl. Acad. Sci. USA* **92**:9598–9602.
 26. Dunfield, P. F., W. Liesack, T. Henckel, R. Knowles, and R. Conrad. 1999. High-affinity methane oxidation by a soil enrichment culture containing a type II methanotroph. *Appl. Environ. Microbiol.* **65**:1009–1014.
 27. Felzenberg, E. R., G. A. Yang, J. G. Hagenzielen, and J. S. Poindexter. 1996. Physiological, morphologic and behavioral response of perpetual cultures of *Caulobacter crescentus* to carbon, nitrogen and phosphorus limitations. *J. Ind. Microbiol.* **17**:235–252.
 28. Fujiwara, Y., K. Takai, K. Uematsu, S. Tsuchida, J. C. Hunt, and J. Hashimoto. 2000. Phylogenetic characterization of endosymbionts in three hydrothermal vent mussels: influence on host distributions. *Mar. Ecol. Prog. Ser.* **208**:147–155.
 29. Fuse, H., M. Ohta, O. Takimura, K. Murakami, H. Inoue, Y. Yamaoka, J. M. Oclarit, and T. Omori. 1998. Oxidation of trichloroethylene and dimethyl sulfide by a marine *Methylococcobium* strain containing soluble methane monooxygenase. *Biosci. Biotechnol. Biochem.* **62**:1925–1931.
 30. Geiselsbrecht, A. D., B. P. Hedlund, M. A. Tichi, and J. T. Staley. 1998. Isolation of marine polycyclic aromatic hydrocarbon (PAH)-degrading *Cyclocasticus* strains from the Gulf of Mexico and comparison of their PAH degradation ability with that of Puget Sound *Cyclocasticus* strains. *Appl. Environ. Microbiol.* **64**:4703–4710.
 31. Grosse, S., L. Laramée, K. D. Wendlandt, I. R. McDonald, C. B. Miguez, and H. P. Kleber. 1999. Purification and characterization of the soluble methane monooxygenase of the type II methanotrophic bacterium *Methylocystis* sp. strain WI 14. *Appl. Environ. Microbiol.* **65**:3929–3935.
 32. Gullede, J., and C. M. Cavanaugh. 1999. Empirical T_d determination for oligonucleotide probes using a PCR thermal cycler. *BioTechniques* **27**:666–670.
 33. Hanada, S., T. Shigematsu, K. Shibuya, M. Eguchi, T. Hasegawa, Y. Suda, Y. Kamagata, T. Kanagawa, and R. Kurane. 1998. Phylogenetic analysis of trichloroethylene-degrading bacteria newly isolated from soil polluted with this contaminant. *J. Ferment. Bioeng.* **86**:539–544.
 34. Hanson, R. S., and T. E. Hanson. 1996. Methanotrophic bacteria. *Microbiol. Rev.* **60**:439–471.
 35. Hanson, R. S., A. I. Netrusov, and K. Tsuji. 1992. The obligate methanotrophic bacteria *Methylococcus*, *Methylomonas*, and *Methylosinus*, p. 2350–2364. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), *The Prokaryotes*, vol. III. Springer-Verlag, New York, N.Y.
 36. Henckel, T., M. Friedrich, and R. Conrad. 1999. Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase. *Appl. Environ. Microbiol.* **65**:1980–1990.
 37. Holmes, A. J., N. J. Owens, and J. C. Murrell. 1995. Detection of novel marine methanotrophs using phylogenetic and functional gene probes after methane enrichment. *Microbiology* **141**:1947–1955.
 38. Kalyuzhnaya, M. G., V. N. Khmelenina, S. Kotelnikova, L. Holmquist, K. Pedersen, and Y. A. Trotsenko. 1999. *Methylomonas scandinavica* sp. nov., a new methanotrophic psychrotrophic bacterium isolated from deep igneous rock ground water of Sweden. *Syst. Appl. Microbiol.* **22**:565–572.
 39. Kalyuzhnaya, M. G., V. N. Khmelenina, N. E. Suzina, A. M. Lysenko, and Y. A. Trotsenko. 1999. New methanotrophic isolates from soda lakes of the southern Transbaikalian region. *Mikrobiologiya* **68**:592–600.
 40. Khmelenina, V. N., M. G. Kalyuzhnaya, N. G. Starostina, N. E. Suzina, and Y. A. Trotsenko. 1997. Isolation and characterization of halotolerant alkaliophilic methanotrophic bacteria from Tuva soda lakes. *Curr. Microbiol.* **35**:257–261.
 41. Krueger, D. M., and C. M. Cavanaugh. 1997. Phylogenetic diversity of bacterial symbionts of *Solemya* hosts based on comparative sequence analysis of 16S rRNA genes. *Appl. Environ. Microbiol.* **63**:91–98.
 42. Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Parker, Jr., P. R. Saxman, J. M. Stredwick, G. M. Garrity, B. Li, G. J. Olsen, S. Pramanik, T. M. Schmidt, and J. M. Tiedje. 2000. The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res.* **28**:173–174.
 43. Mandernack, K. W., C. A. Kinney, D. Coleman, Y. S. Huang, K. H. Freeman, and J. Bogner. 2000. The biogeochemical controls of N_2O production and emission in landfill cover soils: the role of methanotrophs in the nitrogen cycle. *Environ. Microbiol.* **2**:298–309.
 44. McDonald, I. R., G. H. Hall, R. W. Pickup, and J. C. Murrell. 1996. Methane oxidation potentials and preliminary analysis of methanotrophs in a blanket peat bog using molecular ecology techniques. *FEMS Microbiol. Ecol.* **21**:197–211.
 45. McDonald, I. R., H. Uchiyama, S. Kambe, O. Yagi, and J. C. Murrell. 1997. The soluble methane monooxygenase gene cluster of the trichloroethylene-degrading methanotroph *Methylocystis* sp. strain M. *Appl. Environ. Microbiol.* **63**:1898–1904.
 46. Murrell, J. C., I. R. McDonald, and D. G. Bourne. 1998. Molecular methods for the study of methanotrophic ecology. *FEMS Microbiol. Ecol.* **27**:103–114.
 47. Neidhardt, F. C., R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umberger (ed.). 1996. *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
 48. Polz, M. F., and C. M. Cavanaugh. 1997. A simple method for quantification of uncultured microorganisms in the environment based on in vitro transcription of 16S rRNA. *Appl. Environ. Microbiol.* **63**:1028–1033.
 49. Raskin, L., J. M. Stromley, B. E. Rittmann, and D. A. Stahl. 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Appl. Environ. Microbiol.* **60**:1232–1240.
 50. Reeburgh, W. S., S. C. Whalen, and M. J. Alperin. 1993. The role of methylo-trophy in the global methane budget, p. 1–14. In J. C. Murrell and D. P. Kelly (ed.), *Microbial growth on C_1 compounds*. Intercept, Andover, United Kingdom.
 51. Ren, T., R. Roy, and R. Knowles. 2000. Production and consumption of nitric

- oxide by three methanotrophic bacteria. *Appl. Environ. Microbiol.* **66**:3891–3897.
52. **Ross, J. L., P. I. Boon, P. Ford, and B. T. Hart.** 1997. Detection and quantification with 16S rRNA probes of planktonic methylophilic bacteria in a floodplain lake. *Microb. Ecol.* **34**:97–108.
53. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
54. **Stahl, D. A., and R. Amann.** 1991. Development and application of nucleic acid probes, p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.), *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons, New York, N.Y.
55. **Tourova, T. P., M. V. Omel'chenko, K. V. Fegeding, and L. V. Vasil'eva.** 1999. The phylogenetic position of *Methylobacter psychrophilus* sp. nov. *Mikrobiologiya* **68**:493–495.
56. **Tsien, H. C., B. J. Bratina, K. Tsuji, and R. S. Hanson.** 1990. Use of oligodeoxynucleotide signature probes for identification of physiological groups of methylophilic bacteria. *Appl. Environ. Microbiol.* **56**:2858–2865.
57. **Tsuji, K., H. C. Tsien, R. S. Hanson, S. R. DePalma, R. Scholtz, and S. LaRoche.** 1990. 16S ribosomal RNA sequence analysis for determination of phylogenetic relationship among methylophilic bacteria. *J. Gen. Microbiol.* **136**:1–10.
58. **Wise, M. G., J. V. McArthur, and L. J. Shimkets.** 2001. *Methylosarcina fibrata* gen. nov., sp. nov. and *Methylosarcina quisquiliarum* sp. nov., novel type 1 methanotrophs. *Int. J. Syst. Evol. Microbiol.* **51**:611–621.
59. **Wise, M. G., J. V. McArthur, and L. J. Shimkets.** 1999. Methanotroph diversity in landfill soil: isolation of novel type I and type II methanotrophs whose presence was suggested by culture-independent 16S ribosomal DNA analysis. *Appl. Environ. Microbiol.* **65**:4887–4897.